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(54) Title: TRANSFORMATION OF MONOCOT CELLS

(57) Abstract

A process for genetically transforming the nuclear genome of a cell of a monocotyledonous plant, particularly a gramineous plant, comprising the step of: electroporating, with one or more DNA fragments, either a culture of suspended cells of the plant or cells of the plant capable of forming the culture, each cell possessing at least part of its plant cell wall.

TRANSFORMATION OF MONOCOT CELLS

This invention relates to a rapid and efficient method for transforming walled cells of monocotyledonous plants, especially gramineous plants, particularly rice, wheat, corn, barley and other major cereals.

This invention also relates to novel transgenic monocotyledonous plants, particularly gramineous plants, obtainable by this method.

Background of the Invention

In recent years, there has been a tremendous expansion of the capabilities for the genetic engineering of plants. Transgenic plants of many dicotyledonous plant species have been produced. However, many species of plants, especially those belonging to the Monocotyledonae and particularly the Gramineae including economically important species such as corn, wheat and rice, have proven to be very recalcitrant to stable genetic transformation.

Difficulties that have been encountered have resided principally in the inability to combine integrative transformation of monocot plant cells (i.e., the stable insertion of foreign DNA in the nuclear genome of the plant cells) with the regeneration of fertile adult plants from those transformed cells. It has been suggested that such difficulties have been predominantly due to the nonavailability of cells that are competent with respect to 1) DNA uptake, 2) integration of DNA in the genome, and 3) regeneration capability (Potrykus I. (1990) Bio/Technology 9:535). Various methods used to transform cereals have been reviewed in the light of the criteria necessary to assess the stable transformation (Potrykus I. (1990) Bio/Technology 9:535; Potrykus (1991) Annu.Rev.Plant Physi l. Plant M l. Biol. 42:205). In g n ral, direct g n transfer into protoplasts (by polyethyl neglycol (PEG)

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treatment and/or electroporation) seems to have had the best potential but has nevertheless been hampered by the fact that regeneration from protoplasts has been difficult to achieve for most genotypes. In practice, protoplasts have most often been obtained from cell suspension cultures (Lazzeri and Lörz (1988) Advances in Cell Culture Vol.6, Academic press, p. 291; Ozias-Akins and Lörz (1984) Trends in Biotechnology 2:119, Hodges et al (1991) In "Rice Biotechnology" ed. Khush and Toenniessen, C.A.B. International, United Kingdom, p. 157; Lynch et al, (1991) In "Rice Biotechnology" ed. Khush and Toenniessen, C.A.B. International, United Kingdom, p. 135).

As plant regeneration from protoplasts has generally been limited to a relatively small number of genotypes for various species, it has been difficult to develop a generally effective protoplast-based procedure. Therefore, other approaches have recently been explored, particularly in rice.

Lee et al (1991) Proc. Natl. Acad. Sci. USA ("PNAS") 88:6389 have reported the PEG-mediated transformation of small rice cell groups, obtained from rice suspension cultures. Plantlets that had been stably transformed with the gene encoding ß-glucuronidase (gus) and a gene encoding neomycin phosphotransferase II (neo) could be regenerated.

Christou et al (1991) Biotechnology 9:957 have reported the transformation of cells of immature zygotic embryos by bombarding the embryos with DNA-coated gold particles. Transgenic rice plants containing the gus gene with either a gene conferring resistance to phosphinothricin (bar) or to hygromycin (hyg) could be regenerated. The introduced genes were reported to segregate in a normal Mendelian ratio in the progeny.

Intr duction of DNA into intact plant c lls by m ans

of electroporation – a process that is often referred to as electroinjection (see review by Moriwaka et al (1988). In "Biotechnology in Agriculture", Alan R. Liss, Inc. pp. 175-

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callus), from which phenotypically normal (e.g., fertile) plants can be regenerated either by means of organogenesis or, preferably, embryogenesis. Thereby, the resulting transformed cells of this invention can be grown into callus, and plants, preferably phenotypically normal plants, which stably possess and express the one or more genes of interest located on the one or more DNA fragments, can then be regenerated. Such regenerated plants (particularly barley, rice and wheat), as well as their offspring, their seeds, and their transformed cells also form part of this invention.

Detailed Description of the Invention

In carrying out the method of this invention, cultures of suspended plant cells having at least part of their plant cell walls, such as cell suspension cultures, can be obtained from monocotyledonous plants in a conventional manner (Li et al (1990) Plant Mol. Biol. Rep. 8:276; Wen et al (1991) Plant Mol. Biol. Rep. 9:308; Hodges et al (1991) In "Rice Biotechnology" ed. Khush and Toenniessen, C.A.B. International, United Kingdom, p. 157; Yang et al (1991) Aust.J.Plant Physiol. 18:445; Redway et al (1990) Plant Cell Reports 8:714; Jähne et al (1991) Theor.Appl.Genet. 82:74; Gordon-Kamm et al (1990) The Plant Cell 2:603; Fromm et al (1990) Bio/Technology 8:833; Rhodes et al (1988) Bio/Technology 6:56; Vasil and Vasil (1986) J.Plant Physiol. 124:399; Kamo and Hodges (1986) Plant Science 45:111). Since cell suspension cultures have typically been generated so as to provide protoplasts, which can then be transformed and cultured to produce transgenic plants, procedures for making such cell cultures have generally b en directed towards establishing and maintaining regenerabl suspension cultures (i.e., cell suspension

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cultures from which regenerable callus can be obtained).
However, since regeneration in cereals occurs mainly by
means of embryogenesis, cell suspension cultures of
cereals, from which regenerable (in this case, embryogenic)
callus can be obtained, will generally be embryogenic
suspension cultures. Hence, in the following Description
and Examples, the method of this invention is described
mainly with reference to embryogenic suspension cultures of
cereals, such as rice, as a starting material. However, the
method of the invention can be applied to any culture of
suspended walled cells obtained from any monocotyledonous
plant species, particularly to any culture of regenerable
suspended cells, including any culture of cells regenerable
by organogenesis, as well as to walled monocotyledonous
cells which can be used to form such cultures of suspended
walled cells. Preferred cultures of suspended cells are the
various types of liquid cultures which can be obtained by
conventional methods, in the course of establishing cell
suspension cultures, and which are characterized by:
initially, suspended callus clumps, and later,
progressively more homogeneous suspended cell aggregates in
liquid culture media. The method of this invention is
equally applicable to the initial and the later stages but
will be particularly exemplified with respect to preferred
cultures of suspended cell clumps or aggregates.

Whether a culture of suspended walled cells, such as
a culture of suspended cell clumps or a cell suspension
culture (e.g., an embryogenic suspension culture), of a
particular line of a monocot species (e.g., rice) is
suitable for plant regeneration can be determined by
plating a large number (i.e., at least 100) of cell
aggregat s derived from the suspension (r calli deriv d
from such c ll aggr gates n a suitable propagation medium)

on a suitable regeneration medium and determining what
proportion of the aggregates give rise to phenotypically
normal, fertile plants. If normal fertile plants are
obtained from at least about 10%, preferably at least 25%,
particularly at least 50%, of the cell aggregates, the
suspension culture can be considered to be suitable for the
purposes of using the method of this invention to obtain
transgenic monocotyledonous plants.

Embryogenic suspension cultures of this invention can
be established and maintained by conventional procedures.
The embryogenic suspension cultures can generally be
described as fast growing and homogeneous in cell type.
They consist of well-dispersed aggregates, which are
composed of a few to approximately 200 tightly packed
embryogenic cells, in liquid (e.g., aqueous) medium. The
embryogenic cells are round- or oval-shaped, actively
dividing and rich in cytoplasm, they can contain lipid
droplets and starch grains, and they retain at least part,
preferably all, of their cell walls. The embryogenic cells
can have doubling times of, for example, 27 to 32 hours
and, after plating on suitable media, can give rise to
embryogenic calli, from which plants can be regenerated.
The specific appearance and characteristics of a given
regenerable suspension culture, for example the size of
cell clusters, the growth rate, or the color, and the time
required for establishing the suspension culture, may
depend on the plant species and cultivar used, on the
media, and on the physical culture conditions. Cultures of
suspended cells that are not cell suspension cultures
(i. e., cultures of suspended cell clumps) will generally
consist essentially of cell aggregates that 1) are
r latively m re heterogen us in siz (and th cultures may
 ven contain a large numb r f relatively large cell

clumps) and cell type, and 2) contain cells that readily
divide and generally do not show signs of necrosis (e. g.,
browning). Procedures that can be used for establishing and
maintaining embryogenic suspension cultures have been
described, for example, for: rice (Li et al (1990) Plant
Mol. Biol. Rep. 8:276; Wen et al (1991) Plant Mol. Biol.
Rep. 9:308; Hodges et al (1991) In "Rice Biotechnology" ed.
Khush and Toenniessen, C.A.B. International, United
Kingdom, p. 157), wheat (Yang et al (1991) Aust.J.Plant
Physiol. 18:445; Redway et al (1990) Plant Cell Reports
8:714), barley (Jähne et al (1991) Theor.Appl.Genet.
82:74), and corn (Gordon-Kamm et al (1990) The Plant Cell
2:603; Fromm et al (1990) Bio/Technology 8:833; Rhodes et
al (1988) Bio/Technology 6:56; Vasil and Vasil (1986)
J.Plant Physiol. 124:399; Kamo and Hodges (1986) Plant
Science 45:111).

    Likewise, general procedures for establishing
cultures, especially regenerable cultures, of suspended
cells, particularly of cell clumps, of this invention are
well known to those skilled in the art. In fact, media and
procedures that are conventionally used during the
establishment of cell suspension cultures can generally be
used for the establishment of such cultures of suspended
cells, irrespective of whether cell suspension cultures
could be obtained from them. In fact, it is believed that
the establishment of such a culture of suspended cells is
generally easier than the establishment of a cell
suspension culture.

    Explants that can be used to induce callus, from which
suitable cultures of suspended cells of this invention can
be obtained, are well known. For rice, for example, such
explants include dry seeds, immature embryos, young leaf
bases, immature infl r sc nses, anthers, microspores, nod s

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and roots (particularly root tips), but for other cereals, some of the above explants cannot be used as effectively. It is generally believed that immature embryos are the preferred explants for the induction of callus, particularly regenerable callus. It is believed, however, that those skilled in the art will generally be able to modify and optimize existent media and procedures for use with particular plant species or for particular lines and genotypes within a plant species.

For the purposes of this invention, particularly where the transformed cells are to be regenerated into transgenic plants, it is preferred that the suspension cultures be relatively young, preferably not older than about four months, especially not older than three months, particularly for suspensions of rice cells. Thus, it is often preferred that electroporation of suspended cells, as described below, be carried out before the fourth month, preferably before the third month, after initiation of the suspension culture. It is also preferred that the majority of the cells of the embryogenic suspension culture have a chromosome number that is normal for the plant species, from which the culture is derived. In this respect, it is preferred that at least about 50%, preferably at least 75%, particularly 80%, quite particularly 90%, of such cells have a normal chromosome number. Hence, cultures of suspended cells are preferably used which are significantly younger than established cell suspension cultures in order to reduce significantly any somaclonal variations and other adverse effects in plants that are regenerated from such cells.

The present invention is based on the surprising finding that cultures of suspended walled monocotyledonous cells, particularly cultures of such cells from which

regenerable (e.g., embryogenic) callus can be obtained, especially relatively young embryogenic suspension cultures of such cells, as well as walled monocotyledonous cells capable of being used to form such cultures of suspended cells, are competent, not only with respect to regeneration of phenotypically normal plants, but also with respect to DNA uptake by means of electroporation and to subsequent integrative transformation.

The walled monocot cells, to be transformed by the method of this invention, will usually be part of cell aggregates. Whenever such cell aggregates are obtained from cell suspension cultures, particularly embryogenic cell suspension cultures, such cell aggregates will consist of a few to several hundred (i.e., up to about 500) cells and will generally have an average diameter that is smaller than about 0.5 mm. When such cell aggregates are obtained from tissue or callus, preferably regenerable callus, that can be used to form a liquid culture of cell aggregates, they will usually be much larger, with an average diameter of between about 0.5 and 3 mm, preferably with an average diameter of between 1 and 2 mm. When such cell aggregates are obtained from a liquid culture of suspended cells such as from a culture obtained during the establishment of a cell supension culture, they will generally be rather heterogeneous in size, with average dimensions between about 0.5 and 3 mm. However, the dimensions of cell aggregates, described above and in the Examples, are considered preferred dimensions in view of the dimensions of the electroporation cuvettes, which are described below, and are not necessary dimensions for this invention.

In accordance with this invention, electroporation can be carri d out in a conv nti nal manner (s , e.g., Fromm t al (1987) Meth. Enzymol. 153:351). In this r gard,

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walled cells, particularly aggregates of walled cells such
as are contained in a culture of suspended cells (e.g., an
embryogenic suspension culture) or such as can be used to
form such a culture, can be transferred to a cuvette
suitable for use with an electroporation apparatus (e.g.,
as described by Dekeyser et al (1990) The Plant Cell
$\underline{2}$:591). Alternatively, the walled cells, particularly
aggregates thereof, can be suspended in electroporation
buffer and transferred by pipette to the cuvette, or the
liquid medium can be removed from a suspension culture and
its walled cells can then be transferred by spatula to
cuvettes that already contain a suitable volume of
electroporation buffer. Preferably, about 30 mg to 150 mg,
particularly 50 mg to 125 mg, most particularly 75 mg to
100 mg, of cell aggregates per 100 to 200 μl, preferably
100 to 150 μl, of electroporation buffer are transferred to
the cuvette.

Prior to transfer to the cuvettes, it is preferred
that the walled cell aggregates be suspended in the
electroporation buffer, preferably while shaking, for a
period of about 15 minutes to 3 hours, preferably for a
period of about 45 minutes to 1.5 hours, but the period can
be decreased down to a few (i. e., 1 to 5) minutes. Also,
the incubation of a cell material need not be carried out
in the electroporation buffer but can in fact be carried
out in any hypertonic buffer.

Prior to electroporation, it may also be desirable to
treat briefly the cell aggregates with plant cell wall-
degrading enzymes or with mechanical forces (such as
sieving through a fine mesh) in order to damage slightly
the cell walls or to make the cell aggregates more
h mogenous in size. When us d, such an nzyme pretreatment
should pr ferably not be for long r than 30 minutes,

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particularly not for longer than 10 minutes, quite particularly not for longer than 3 to 5 minutes. Enzymes or enzyme compositions that can be used for this purpose are well known (see, e.g., Power and Chapman (1985) In "Plant Cell tissue Culture: A Practical Approach", IRL Press, Oxford).

After the DNA fragments are added to the cuvette containing the walled cells, particularly aggregates thereof, in electroporation buffer, the electroporation can be carried out in accordance with this invention. Preferably, the DNA is coincubated for as long as about two or three hours or as little as about five to fifteen minutes (and as low as about one minute), but typically for about one hour, with the walled cells prior to electroporation. It is believed that best results can be obtained with linear, rather than circular, DNA of relatively small size, preferably smaller than about 20 kb, especially smaller than 15 kb, particularly smaller than 10 kb, quite particularly smaller than 6 kb (e.g., down to about 2-3 kb). In this regard, multiple linear DNA fragments of different composition can be used to transform the competent monocot plant cells of this invention with multiple genes of interests. Preferably, about 5 to 30 μg, particularly about 10 to 25 μg, quite particularly about 10 or 20 μg, of DNA are added to the cuvette containing the cell aggregates. Substances that prevent DNA degradation, such as spemidine, can be added.

Particular electroporation conditions are not believed to be critical, and good results can be obtained (e.g., in rice) with one pulse with an electrical field strength of between about 600 and 700 V/cm discharged from a capacitor f about 800 to 900 μF. Although optimal electroporation conditi ns f r different types of c lls and th ir

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aggregates (e.g., from suspension cultures) are likely to be different, conditions as described by Fromm et al (1987) Meth. Enzymol. 153:351 and Dekeyser et al (1990) supra can generally be used. In this regard, optimal electroporation conditions for any type of cell aggregate are believed to be dependent on the plant species, being transformed and, when using suspension cultures, the age and general condition of the suspension, and such conditions can be experimentally determined. Hence, it is generally preferred that an exploratory experiment be carried out initially with the cell aggregates, in which experiment no DNA is added to the electroporation cuvette containing the cell aggregates in electroporation buffer and that, after the electroporation pulse, at least about 50%, preferably at least 75%, particularly at least 90%, of the cell aggregates develop into calli after plating on solid culture medium.

The composition of the electroporation buffer is also not believed to be critical, and generally, conventional electroporation buffers can be used (see, e.g., Fromm et al (1987) supra).

When the transformation by electroporation is completed, the cell aggregates, containing the transformed monocot cells, are transferred to a suitable culture medium (which may be a solid medium, a bead-type medium, or even a liquid medium), preferably a selective medium when the transformed cells contain DNA fragments encoding a selectable marker. This transfer should be as soon as possible after, preferably immediately after, the transformation event and especially within about one to three days after the transformation event.

Preferably, cell aggregates transformed with DNA fragments encoding a selectable marker are cultured using

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conventional culture conditions, culture procedures, and culture media (see, e.g., references in Vasil (1988) supra) supplemented with a selective agent. The selection of the selective agent will depend on the selectable marker used in the DNA fragments to transform the walled cells, as discussed below. The concentration of the selective agent should provide a suitable selective pressure on the transformed cells so that only stably transformed cells, in which the DNA fragments encoding the selectable marker are integrated, preferably fully integrated, in the genome of the cells, survive and can be isolated. Although such transformed cell aggregates can be cultured for a few days on non-selective medium, it is preferred that they be transferred to selective medium as soon as possible and maintained for a sufficiently long period (e.g., as long as about six months), preferably at least about one month, especially two to three months, to produce significant amounts of transformed morphogenic callus, such as transformed embryogenic callus, which can be used to regenerate a phenotypically normal plant. It is also preferred that the hypertonicity of the medium be maintained for a limited time (e.g., up to about two to three weeks), for instance by supplementing the medium with mannitol.

In accordance with this invention, any DNA fragment can be integrated in the genome, particularly the nuclear genome, of a monocotyledonous plant. Generally, the DNA fragment contains a foreign or endogenous gene or other DNA sequence which is functional in the transformed plant cells and confers an additional property to such cells and to plants regenerated from the cells. To this end, the DNA fragment preferably comprises one or more chimaeric genes which c ntain th following perably linked DNA sequ nces:

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1) a promoter sequence capable of directing expression of a coding sequence in the plant cell (a "promoter"); 2) a sequence (a "coding sequence") coding for a protein with a specific activity within the plant cell (a "protein of interest"); and 3) suitable 3' transcription regulation signals. In order to obtain the required functionality of the protein, it may also be necessary that the protein be targeted to one or more particular compartments of the plant cell, such as the cytosol, mitochondria, chloroplasts or endoplasmatic reticulum. For targeting to the cytosol, the chimaeric gene(s), as described above, can be used as such. However for targeting to the other compartments, it is required that there be an additional sequence (a "targeting sequence") between the DNA sequences 1) and 2) of the chimaeric gene(s). If required, the chimaeric gene(s) can also contain transcriptional and/or translational enhancers, and the codon usage of the DNA sequences can be optimized for expression in plant cells.

Chimaeric genes in accordance with this invention can be constructed according to well-established principles and techniques. In this regard, the various DNA sequences should be linked so that translation is initiated at the initiation codon of the coding sequence of the protein (or of the targeting sequence, when present).

It is believed that the various constitutive and organ- and tissue-specific promoters that are presently used to direct expression of genes in transformed dicotyledonous plants will also be suitable for use in transformed monocots of this invention. In this regard, particular plant cells can be transformed with a chimaeric gene comprising: a coding sequence encoding a protein of int r st; and upstream (i.e., 5') ther f, either a foreign r an endogen us promoter suitabl for expression f th

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coding sequence. Suitable foreign constitutive promoters include: the promoter of the cauliflower mosaic virus ("CaMV") isolates CM1841 (Gardner et al (1981) Nucl. Acids. Res. 9:2871) and CabbB-S (Franck et al (1980) Cell, 21:285) (the "35S promoter") which directs constitutive expression of heterologous genes (Odell et al (1983) Nature 313:810); a related promoter (the "35S3 promoter") which can be isolated from the CaMV isolate CabbB-JI (Hull and Howell (1978) Virology 86:482) and which differs from the 35S promoter in its sequence (the sequence of the 35S3 promoter is disclosed in European patent publication ("EP") 359617) and in its greater activity in transgenic plants (Harpster et al (1988) Mol. Gen. Genet. 212:182); and the TR1' and the TR2' promoters which drive the expression of the 1' and 2' genes, respectively, of the T-DNA of _Agrobacterium_ (Velten et al (1984) EMBO J. 3:2723) and are wound-induced promoters. Suitable organ-specific, tissue-specific and/or inducible foreign promoters are also known (see, e.g., references cited in Kuhlemeier et al (1987) Ann. Rev. Plant Physiol. 38:221) such as the promoters of the small subunit genes (such as the 1A gene) of 1,5-ribulose bisphosphate carboxylase of _Arabidopsis thaliana_ (the "ssu" promoter) which are light inducible promoters (Krebbers et al (1988) Plant Mol. Biol. 11:745) active only in photosynthetic tissue; the anther-specific promoters disclosed in EP 344029; and the seed-specific promoters of, for example, _Arabidopsis thaliana_ (Krebbers et al (1988) Plant Physiol. 87:859). Promoters of particular usefulness for transforming monocots to render them male-sterile, as described in European patent publication ("EP") 344029, are the tapetum-specific promoters PTA29, PTA26 and PTA13, particularly PTA29, of EP 344029.

Likewise, it is believed that kn wn 3' transcription

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regulation sequences and polyadenylation signals used in transformed dicotyledonous plants can be used in transformed monocots of this invention. Such 3' transcription regulation signals can be provided downstream (i.e. 3') of the coding sequence. In this regard, a particular plant cell can be transformed with a chimaeric gene containing either foreign or endogenous, transcription termination and polyadenylation signals suitable for obtaining expression of the chimaeric gene. For example, the foreign 3' untranslated ends of genes, such as gene 7 (Velten and Schell (1985) Nucl. Acids Res. 13:6998), the octopine synthase gene (Gielen et al (1983) EMBO J. 3:835) and the nopaline synthase gene of the T-DNA region of Agrobacterium tumefaciens Ti-plasmid can be used.

For construction of a chimaeric gene which can be expressed in a transformed plant cell, preferably in its cytoplasm followed by translocation of its protein of interest to the cell's mitochondria, chloroplasts and/or lumen of the endoplasmatic reticulum, suitable targeting sequences are known. Selection of such targeting sequences is not believed to be critical, and a particular plant cell can be transformed with a chimaeric gene containing either a foreign or endogenous targeting sequence encoding a targeting peptide which will provide translocation of the expression product of the gene. By "targeting peptide" is meant a polypeptide fragment which is normally associated, in an eucaryotic cell, with a chloroplast or mitochondrial protein or subunit of the protein or with a protein translocated to the endoplasmatic reticulum and which is produced in a cell as part of precursor protein encoded by the nuclear DNA of the cell. The targeting peptide is responsible for the translocation process of the nuclear-encoded chloroplast r mit chondrial protein or subunit

into the chloroplast or the mitochondria or the lumen of
the endoplasmatic reticulum. During the translocation
process, the targeting peptide is separated or
proteolytically removed from the protein or subunit. A
targeting sequence can be provided in the chimaeric gene to
express a targeting peptide which can translocate an
expressed protein of interest within a transformed plant
cell as generally described in European patent applications
("EPA") 85402596.2 and 88402222.9. A suitable targeting
peptide for transport into chloroplasts is the transit
peptide of the small subunit of the enzyme 1,5-ribulose
bisphosphate carboxylase (Krebbers et al (1988) Plant Mol.
Biol. 11:745; EPA 85402596.2), but other chloroplast
transit peptides, such as those listed by Watson (1984)
Nucl. Acids Res. 12:5145 and Von Heijne et al (1991) Plant
Mol. Biol. Rep. 9:104, can also be used. Suitable
mitochondrial targeting peptides are the mitochondrial
transit peptides as described by Schatz (1987) Eur. J.
Biochem. 165:1 and listed by Watson (1984) supra. Suitable
targeting peptides that can translocate a protein of
interest to the lumen of the endoplasmatic reticulum of a
plant cell are, for instance, the signal peptides described
by Von Heijne (1988) Biochem. Biophys. Acta 947:307 and
listed by Watson (1984) supra.

Coding sequences that can be used in the production of
transgenic dicotyledonous plants are well known (see, for
example, the coding sequences listed in Weising et al
(1988) Annual Rev. Genet. 22:421), and it is believed that
such coding sequences can be put to equally good use in
transformed monocotyledonous plants in accordance with this
invention. In this respect, the coding sequences can be
either for ign or endogenous to the plants and can, for
xampl , cod for pr teins which: are t xic to insect

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species, thus protecting the plants against insect attack (EP 193259, EP 305275 and EP 358557); protect the plants against stress conditions (EP 359617); confer on the plants a resistance or tolerance to specific herbicides (EP 242236); kill or disable plant cells in which the proteins are expressed so that, when the coding sequences are under the control of a male or female organ-specific promoter (EP 344029, WO 92/00274 and WO 92/00275), the proteins can render the plants respectively male sterile (EP 344029) or female sterile (EP 412006); can be extracted from the plants or selected plant organs and optionally be further processed so that the plants can be used as sources of economically important peptides or proteins (EP 319353); or are enriched in nutritionally important amino acids so that transformed plants or their organs, in which the proteins are expressed, can be used as food with enhanced nutritional value for animals or humans (EP 318341).

Coding sequences of particular usefulness for transforming monocots to render them insect-resistant are the genes isolated from Bacillus thuringiensis ("Bt") strains and truncated portions thereof that code for insecticidal crystal proteins and their insecticidal polypeptide toxins (for a review, see: Höfte and Whiteley (1989) Microbiol. Rev. 53:242). The following Bt genes are believed to be particularly important for insect control in cereals (e.g., rice, wheat, corn and barley): the CryIAb gene (EP 193259) and CryIAc gene for control of Helicoverpa species (e.g., H. zea and H. armigera); the CryIAb gene and the CryIb gene (EP 358557) for control of Ostrinia species (e.g., O. nubilalis) in corn; the CryIAc gene for the control of Agrotis species in corn and wheat; and the CryID and CryIE genes (EP 358557) for the control f Spodopt ra species (.g., S. frugiperda) in corn. To achieve

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sufficient expression of such genes in tissues of
transgenic plants, it is preferred that the genes be
modified as described in PCT application PCT/EP 91/00733
(PCT publication WO 91/16432).

Selectable markers in accordance with this invention
can be encoded by chimaeric genes in which the coding
sequences encode proteins which confer on the plant cells,
in which they are expressed, resistance to a selective
agent such as an antibiotic and/or herbicide. Screenable
markers in accordance with this invention can be encoded by
chimaeric genes in which the coding sequences encode
proteins which confer on the plant cells, in which they are
expressed, a different appearance, such as a different
color, making plants transformed with the screenable marker
separable manually. The selection of coding sequences for
a selectable or screenable marker, preferably a selectable
marker, for transforming a monocotyledonous plant in
accordance with this invention is not believed to be
critical, and it is believed that coding sequences for
conventional selectable and screenable markers can be used
(see, for example, the markers listed in Weising et al
(1988) supra). Examples of suitable coding sequences for
selectable markers are: the neo gene (Beck et al (1982)
Gene 19:327) that codes for the enzyme neomycin
phosphotransferase which confers resistance to the
antibiotic kanamycin; the hyg gene (Gritz and Davies (1983)
Gene 25:179) that codes for the enzyme hygromycin
phosphotransferase which confers resistance to the
antibiotic hygromycin; and the bar gene (EP 242236) that
codes for phosphinothricin acetyl transferase which confers
resistance to the herbicidal compounds phosphinothricin and
bialaph s. In using a selectable marker gene coding for a
protein that c nfers toleranc or resistance to a herbicid

or other selective agent that acts on chloroplast
metabolism, such as the bar gene, it is preferred that the
marker gene be part of a chimaeric gene together with a
chloroplast targeting sequence as described above. Examples
of suitable coding sequences for screenable markers are the
gus gene (Jefferson et al (1986) PNAS 6:3901) encoding
beta-glucuronidase and the luciferase gene (Ow et al (1986)
Science 234:856).

During the culturing of transformed cell aggregates of
this invention, the selection pressure, provided by the
presence of a selective agent in the culture media, should
be high enough and should be maintained long enough to
separate transformed cells from untransformed cells. It is
believed, however, that particular selection pressures and
durations are not critical and that the choice of selection
pressures and their durations can be made in a conventional
manner. However, when the bar gene is used as a selectable
marker gene, phosphinothricin (PPT) is preferably used in
concentrations of about 0.5 mg to 50 mg, particularly 2 mg
to 20 mg, per liter of the culture medium.

Morphogenic sectors, preferably embryogenic sectors,
of morphogenic callus, preferably embryogenic callus,
produced in a culture of transformed cells of
electroporated walled cell aggregates (e.g., from
suspension cultures) of this invention, can then be
regenerated into phenotypically normal (e.g., mature and
fertile) plants in a conventional manner (see, e.g.,
references in Vasil (1988) supra, Lazzeri and Lörz (1988)
supra, and Lynch et al (1991) In "Rice Biotechnology" ed.
Khush and Toenniessen, C.A.B. International, United
Kingdom, p. 135 and references cited therein). The
r g nerat d plants, thus obtained, will be transgenic and
will at least c ntain any DNA fragments encoding a

selectable or screenable marker, preferably a selectable marker, stably integrated into their nuclear genome. The presence and expression of other genes of interest can then be evaluated in a conventional manner, such as by means of Southern blotting and/or by the polymerase chain reaction (Sambrook et al (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, N.Y.) and/or by ascertaining the phenotypic expression of the genes of interest.

For the purposes of this invention, a phenotypically normal plant as produced by the transformation and regeneration procedures of this invention should be understood as at least one plant that does not differ substantially from an untransformed plant of the same line in any of its phenotypic characteristics except in those characteristics that are added or changed due to the expression of the DNA fragment(s) introduced in the plant's genome during transformation in accordance with this invention. Of course, any procedure that results in transgenic plants usually produces a number of transgenic plants that display a variety of phenotypes, only some of which are phenotypically normal as defined above.

The method of this invention can be applied to all monocotyledonous plant species, from which liquid cultures of suspended walled cells, particularly liquid cultures of walled cell aggregates, such as suspension cultures, preferably suspension cultures from which regenerable callus, particularly embryogenic callus, can be obtained by in vitro culture of explants derived from various explant sources such as immature and mature zygotic embryos, leaf bases, young inflorescences, anthers, microspores, etc. The method will b especially useful f r th transf rmati n of con mically imp rtant gramin ous crops, particularly the

major cereals, such as rice, wheat, oats, barley, corn, sorghum, rye and millet. The resulting transgenic plants of this invention can be used to create, in a rapid and efficient manner, novel lines and/or cultivars of high agronomic value.

This invention provides a rapid, efficient and reproducible method for transforming walled cells of monocotyledonous plants by: electroporation of cultures of the suspended cells (e.g., cell suspension cultures), as well as walled cells capable of forming such cultures (e.g., cells obtained from explant-derived callus). When regenerable (e.g., embryogenic) suspension cultures of walled cells are electroporated in accordance with this invention, cultures of transformed morphogenic callus can be produced, from which phenotypically normal, fertile plants can be regenerated. This is surprising as electroporation of such walled cells, particularly those of embryogenic suspension cultures, has generally not been regarded as a suitable method for obtaining stable transformants in monocotyledonous plants (see, e.g., Potrykus (1991) Annu. Rev. Plant Physiol. Plant mol. Biol. 42:205). The electroporation of such walled cells, particularly without any enzymatic or mechanical pretreatment thereof, in accordance with this invention is a distinct improvement on existing monocot transformation methods. Because the method of this invention requires only a relatively short period of in vitro culture, the method is far less time and labor consuming than most previous methods. The short tissue culture period also ensures that the occurrence of somaclonal variation is reduced.

The method of this invention can be used to produce novel, phenotypically normal (e.g., fertile), transgenic monocotyledonous plants, particularly gramin ous plants,

quite particularly cereals, most particularly rice, wheat
and barley, which are transformed with at least one (e.g.,
foreign) gene of interest, stably integrated into their
nuclear genome. The method is believed to be relatively
independent of the genotype of the plant, being
transformed, and capable of transforming cells of any
plant, from which regenerable (e.g. embryogenic) suspension
cultures can be obtained from at least one of its tissues.
This makes it possible to transform the majority of monocot
species and a substantial number of lines within each
species. Indeed, the capacity to form suitable regenerable
suspension cultures can be transferred, by means of
classical breeding programs, from one plant line that
posesses such capacity to another line that does not,
making the method of this invention applicable to even more
plant lines.

As described above, stably transformed
monocotyledonous plant cells can be advantageously obtained
by electroporation of cultures of suspended walled plant
cells in accordance with this invention. In this regard, if
regenerable callus (such as embryogenic callus) can be
obtained from the cultures of suspended cells, some of the
so-transformed plant cells of the callus can subsequently
be regenerated in accordance with this invention into
transgenic monocotyledonous plants that contain at least
one gene of interest stably integrated into the genome of
all of its cells.

However, it is believed that a culture of suspended
cells of this invention need not be a "cell suspension
culture" in the strict sense of the term as it is used with
respect to a cell culture to be used for the preparation of
pr toplasts. Indeed, it is b li ved that, in accordance
with this invention, essentially the sam r sults in the

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transformation and regeneration of plant cells can be obtained by electroporation of any culture of plant cells which retain their cell walls and are suspended in a liquid (e.g., aqueous) medium. In this regard, a culture of suspended cells of this invention should be understood as encompassing any liquid culture of cell aggregates obtained from plant tissue or from a callus obtained from plant tissue. In fact, the use of a liquid culture of suspended cell aggregates obtained from callus in the method of this invention could further reduce in vitro culture time and any subsequent somaclonal variation in transformed cells and/or plants. Furthermore, it appears that essentially the same results can be obtained by electroporation of cells from a certain type of callus for each plant species which can be used to form a culture of suspended cells of this invention. For example, in rice, such callus can be part of explant-derived (e.g., embryo-derived) embryogenic callus and consist of compact yellow and/or whitish, often round- or oval-shaped, cell clumps that can be easily separated from the rest of the callus tissue.

The Examples, which follow, illustrate this invention. Unless otherwise indicated, all experimental procedures for manipulating recombinant DNA were carried out by the standardized procedures described in Sambrook et al (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, N.Y. Any oligonucleotides were designed according to the general rules outlined by Kramer and Fritz (1968) Methods in Enzymology 154:350 and synthesized by the phosphoramidite method of Beaucage and Caruthers (1981) Tetrahedron Letters 22:1859 on an Applied Biosystems 380A DNA synthesizer (Applied Biosystems B.V., Maarssen, Netherlands). The compositions f the 2N6, N67, AA, N683, and hormon -fre N6 m dia, used in the Examples, were

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kindly provided by Japan Tobacco Inc., Plant Breeding and Genetics Research Laboratory, 700 Higashibara, Toyoda, Iwata, Shizuoka 438, Japan.

In the following Examples, reference will be made to the following sequence listing:

Sequence Listing

SEQ ID no. 1 -- Sequence of plasmid pDE110 of Example 2.
SEQ ID no. 2 -- Sequence of plasmid pDE4 of Example 4.

Example 1: Establishment and maintenance of rice cell suspension cultures.

Cell suspension cultures of the rice cultivars Nipponbare and Kochihibiki were made from seed-derived callus as follows. Mature dry rice seeds were dehulled, surface sterilized and plated on solid 2N6 medium (N6 medium as described by Chu et al (1975) Sci.Sin.Peking 18:659 supplemented with 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, 1.0 mg/l thiamine HCl, 2.0 mg/l 2,4 dichlorophenoxyacetic acid (2,4-D), 30 g/l sucrose, 2.0 g/l Gelrite, pH 5.8). The plates were incubated at 30°C for 4 weeks, after which, approximately one gram of compact whitish and/or yellow parts of embryo-derived compact and embryogenic callus was transferred to 65 ml AA medium (macronutrients, amino acids, growth regulators and sugar as described for the AA medium of Toriyama and Hinata (1985) Plant Science 41:179 with micronutrients and vitamins from the MS medium as described by Murashige and Skoog (1962) Physiol. Plant. 15:473, pH 5.8) in a 250 ml Erlenmeyer flask. These cultures were maintained in the dark n a rotary shaker at approximately 120 rpm. The cultures were subcultured we kly.

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After the first subculture, all AA medium was removed from the culture flask and replaced by 65 ml fresh AA liquid medium. During subsequent subcultures, 1 to 2 ml of packed cell volumes of smaller, usually creamy or yellow, cell clumps, which were formed when bigger cell clusters dissociated into smaller fragments, were selected and transferred to 65 ml fresh AA medium. At each subculture, care was taken to eliminate cell clusters with brown areas (necrosis). After 1-2 months of subculture, clean (i.e., no brown cell clusters) suspension cultures, consisting of well dispersed and compact cell aggregates of different sizes, were obtained.

The cell suspension cultures of the two cultivars were then maintained by transferring 1 ml packed cell volumes to 65 ml fresh medium during each subculture. The cell clusters of the suspensions were regularly checked for their potential of regenerating plants by plating them on N6S3 medium (N6 medium, but with major salts at half strength, supplemented with 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, 1.0 mg/l thiamine HCl, 876 mg/l glutamine, 266 mg/l aspartic acid, 174 mg/l arginine, 7.5 mg/l glycine, 1.0 g/l casamino acids, 0.2 mg/l naphthaleneacetic acid (NAA), 1.0 mg/l kinetin, 20 g/l sucrose, 4.0 g/l Gelrite, pH 5.8) for plant regeneration.

Example 2: Transformation of rice cell suspension cultures with a herbicide resistance gene.

The cell suspension cultures from rice cultivars Nipponbare and Kochihibiki of Example 1 were transformed with a herbicide resistance gene, and transformed cells were regenerated into transgenic plants as follows.

1. Nipponbare

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A cell suspension culture was established and maintained for a period of two months. Four days after the last subculture, the AA culture medium was removed, the cell clusters were washed with electroporation buffer AA (35 mM L-aspartic acid, 35 mM L-glutamic acid, 5 mM D-gluconic acid, 5 mM 2-[N-morpholino] ethane sulfonic acid (MES), 0.4 M mannitol, pH 5.8 (Tada et al (1990) Theor. Appl. Genet. 80:475)) and kept in this buffer for one hour on a shaker at 30 rpm. Thereafter, the cell aggregates were washed twice with electroporation buffer AA. Approx. 75 mg to 100 mg of cell clusters were transferred to electroporation cuvettes and resuspended in approx. 100 to 150 μl electroporation buffer AA. 15 μg of pDE110 plasmid DNA, linearized with HindIII, were added to each cuvette. Plasmid pDE110 is a plasmid with a length of 4883 bp and contains the phosphinothricin (PPT) resistance gene (bar) under control of the CaMV 35S promoter (EP 359617). The complete sequence of pDE110 is given in SEQ ID no. 1. After addition of the plasmid DNA, the cuvettes were put on ice for 10 min. Then, a single pulse with a field strength of 700 V/cm was discharged from a 800 μF capacitor to the mixture of cell clusters and DNA. Immediately after the pulse, liquid N67 medium (N6 medium supplemented with 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, 1.0 mg/l thiamine HCl, 1.0 mg/l 2,4 D, 0.5 mg/l 6-benzylaminopurine, 20 g/l sucrose, 30 g/l sorbitol, pH 5.8) was added to the cell clusters, which were then plated on to solid selective N67 medium (N6 medium supplemented with 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, 1.0 mg/l thiamine HCl, 1.0 mg/l 2,4-D, 0.5 mg/l 6-benzylaminopurine, 20 g/l sucrose, 30 g/l sorbitol, 2.0 g/l Gelrite, pH 5.8) containing 5 mg/l PPT.

The plat s wer incubat d at 26°C under a light/dark

regime of 16/8 hours. After 6 weeks of culture, PPT-resistant calli developing from the treated suspension aggregates were placed on fresh N67 medium plus PPT for another 12 days. Thereafter, PPT-resistant calli were transferred to plant regeneration medium N6S3 supplemented with 5 mg/l PPT. From two of the selected calli, plants could be regenerated. As soon as the developing plantlets reached a height of approximately 10 cm (usually within one to two months), they were transferred to hormone-free N6 medium (N6 medium supplemented with 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, 1.0 mg/l thiamine HCl, 1.0 g/l casamino acids (vitamin assay), 20 g/l sucrose, 2.0 g/l Gelrite, pH 5.8) and cultured on this medium until they were strong enough to be transferred to soil and to the greenhouse (usually after a period of one to three weeks).

2. Kochihibiki

A cell suspension culture was established and maintained for a period of 4.5 months. Four days after the last subculture, the AA culture medium was removed, the cell clusters were washed with electroporation buffer 9 (0.4 M mannitol, 10 mM KCl, 4 mM $CaCl_2.2H_2O$, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.2) and kept in this buffer on a shaker for one hour. Thereafter, the cell clusters were washed twice with electroporation buffer 9. Approx. 75 to 100 mg of cell aggregates were transferred to electroporation cuvettes and resuspended in approximately 100 to 150 μl electroporation buffer 9 per cuvette. 12 μg of pDE110 plasmid DNA, linearized with EcoRI, were added to each cuvette and coincubated with the cell clusters for 45 min at room temperatur (25° C). The cuvetts were placed on ice for 10 min., and th cell aggregates in each cuvett w re

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electroporated by applying a single pulse discharged from a capacitor with one of the following voltage-capacitance characteristics: 700 V/cm-800 μF (4 cuvettes), 600 V/cm-900 μF (4 cuvettes), 700 V/cm-900 μF (5 cuvettes). Liquid N67 medium was added to each cuvette immediately after the pulse, and the cell clusters were plated on solid selective N67 medium supplemented with 5 mg/l PPT.

The plates were incubated at 26°C under a light/dark regime of 16/8 hours for 19 days. Developing PPT-resistant calli were isolated and transferred to fresh N67 medium plus 5 mg/l PPT and propagated for another 18 days. After this second selection cycle, the well-developing PPT-resistant calli were placed on plant regeneration medium N6S3 supplemented with 5 mg/l PPT. Plants were regenerated from 17 of the selected calli; five calli were from cell aggregates electroporated with a 700 V/cm-800 μF pulse; two calli were from cell aggregates electroporated with a 600 V/cm-900 μF pulse; and ten calli were from cell aggregates electroporated with a 700 V/cm-900 μF pulse. As soon as the developing plantlets reached a height of approximately 10 cm (usually within one to two months), they were transferred to hormone-free N6 medium and cultured on this medium until they were strong enough to be transferred to soil and to the greenhouse (usually after a period of one to three weeks).

In a second round of experiments, the same Kochihibiki suspension culture, described above, was used 5 months after initiation. Five days after the last subculture, the cell clusters were washed in electroporation buffer 9, kept for 45 min. in this buffer on a shaker, and washed twice thereafter with the same buffer. Approximately 75 to 100 mg f cell clusters were transferred to each of 16 electroporation cuvettes and resuspended in approximately

100 to 150 μl electroporation buffer 9 per cuvette. 15 μg
of pDE110 plasmid DNA, linearized with HindIII, were added
to each cuvette. Eight of these cuvettes (batch A) were put
on ice for 10 min and then pulsed; the other eight cuvettes
(batch B) were kept at room temperature for one hour prior
to electroporation. Pulses of 600 V/cm-900 μF (four
cuvettes from each batch) and 700 V/cm-900 μF (four
cuvettes from each batch) were applied to the mixture of
cell clusters and DNA. Immediately after the pulse, N67
liquid medium was added to each cuvette, and the cell
clusters were plated on selective N67 medium supplemented
with 5 mg/l PPT.

     · The plates were incubated as above. After 23 days, the
developing PPT-resistant calli derived from plated
suspension clusters were transferred to fresh selective N67
medium plus 5 mg/l PPT for a second selection cycle and
further propagation. After 21 days, well growing calli were
transferred to plant regeneration medium N6S3 plus 5 mg/l
PPT. Plants could be regenerated from 24 of the selected
calli; two calli were from cell aggregates of batch A
electroporated with a 600 V/cm-900 μF pulse; three calli
were from cell aggregates of batch A electroporated with a
700 V/cm and 900 μF pulse; nine calli were from cell
aggregates of batch B electroporated with a 600 V/cm-900 μF
pulse; and ten calli were from cell aggregates of batch B
electroporated with a 700 V/cm-900 μF pulse. As soon as the
developing plantlets reached a height of approximately 10
cm (usually within one to two months), they were
transferred to hormone-free N6 medium and cultured on this
medium until they were strong enough to be transferred to
soil and to the greenhouse (usually after a period of one
to three weeks).

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Example 3 : Analysis of the transgenic plants from Example 2.

The plants of Example 2 were cultivated in the greenhouse and sprayed with a 0.5% Basta (PPT) solution 4-6 weeks after transfer to soil. All of the plants were Basta-resistant, whereas non-transformed control plants turned brown and died within one week after herbicide treatment.

Southern analysis was performed on 13 selected primary transformants (in vitro plants derived from 13 separate regenerating calli). For Southern analysis, the rice DNA was digested with the restriction enzymes, EcoRV, BglII and PvuII, Southern blotted, and probed with pDE110 DNA. The Southern analysis showed that three of the plants carried single copy inserts of the complete pDE110-derived chimaeric bar gene (i.e., the bar gene with promoter and 3' untranslated end), integrated into the rice genome. Five of the plants had one to three copies of pDE110-derived DNA integrated into their genome, with at least one of the copies containing the complete chimaeric bar gene. Three of the plants carried multiple inserts of pDE110-derived DNA, while two other plants had only parts of the chimaeric bar gene integrated into the genome.

Three of the plants, regenerated from one of two independently transformed calli of Nipponbare of Example 2.1, were grown to maturity and set seed. One of these plants was analyzed in detail. Southern analysis of this plant proved that its genome contained an insert, at a single locus in the rice genome, that comprises one almost complete copy of the transforming DNA (pDE110), including the P35S-bar-3'nos chimeric gene. From this plant (designated as E253), 81 seeds (after selfing) were harvsted. Some f the s eds were used for an analysis of

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the segregation of the <u>bar</u> gene in the progeny plants. 93
seedlings were sprayed with Basta: 73/93 seedlings were
Basta resistant, 20/93 seedlings were Basta sensitive (x^2=
0.52, which is not significantly different from Mendelian
segregation at a single dominant locus). Four of the Basta-
resistant progeny plants were analyzed in Southern blots.
All four plants had the same hybridization pattern as
determined for primary transformant E253.

For Kochihibiki, integration of the transforming DNA
was confirmed by Southern analysis of <u>in vitro</u> shoots or
regenerated plants obtained from various calli. One
regenerated plant (designated as K32), obtained from one of
the 24 independently transformed calli of Example 2.2 was
analyzed in more detail and was shown to contain an almost
complete copy of the transforming DNA (pDE110), including
the complete P35S-<u>bar</u>-3'nos chimeric gene. From primary
transformant K32, the 81 seeds were harvested, and
segregation of the <u>bar</u> gene was analyzed in 98 progeny
plants by Basta spraying: 80/98 seedlings were Basta-
resistant, 18/98 seedlings were Basta-sensitive (x^2= 2.36,
which is not significantly different from Mendelian
segregation at a single dominant locus). Four of the Basta-
resistant progeny plants were analyzed in Southern blots.
All four plants had the same hybridization pattern as
determined for primary transformant K32.

<u>Example 4: Transformation of seed-derived callus cells</u>

Mature dry seeds of the rice cultivars <u>Kochihibiki</u> and
<u>Chiyonishiki</u> were dehulled, surface sterilized and plated
on solid 2N6 medium. The plates were incubated at 30°C for
approximately 1 month. Embryogenic suspension cultures were
then initiated using the small, round- and oval-shaped,
yellow and whitish callus clumps (some of which hav

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globular structures on the surface which may represent
proembryos) that appeared in the resulting callus and that
constituted the majority of the observed callus types in
the culture or were located at the surface of larger calli.
These callus clumps were usually not attached to the
surfaces of the larger calli or to each other, and they
were easily removed individually with a pair of forceps.

The callus clumps, with an average maximum diameter of
about 2 mm, were carefully transferred immediately to
electroporation buffer 9 and kept in this buffer for one
hour on a shaker (30 rpm). Thereafter, the callus clumps
were washed once with electroporation buffer 9. Approx. 75
to 100 mg of callus clumps were transferred to
electroporation cuvettes and resuspended in approx. 150 μl
buffer 9. 10 μg of pDE4 plasmid DNA were added to each
cuvette. Plasmid pDE4 is a plasmid with a length of 5642 bp
and contains a gene (gus) encoding beta-glucuronidase
(Jefferson et al (1986) PNAS 83:8447) under the control of
the CaMV 35S3 promoter (EP 359617). The complete sequence
of pDE4 is given in SEQ ID no. 2. The plasmid DNA was
coincubated with the callus clumps for 45 min. at room
temperature. The cuvettes were then placed on ice for 10
min. Thereafter, a single pulse with a field strength of
600 V/cm was discharged from a 900 μF capacitor to the
mixture of callus clumps and DNA. Immediately after the
pulse, liquid AA medium was added to each cuvette, and the
callus clumps were transferred to a petri dish (3.5 mm
diameter). The liquid was removed and replaced by 2 ml AA
medium per petri dish. The callus clumps were cultured in
the dark for 5 days. Thereafter, the callus clumps were
transferred   to   X-gluc   (5-bromo-4-chloro-3-indolyl
glucur nid) s lution for in situ detection of beta-
glucuronidase (GUS) activity (as described in Deneck et al

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(1989) Methods in Mol. and Cell. Biol., Jan/Feb 1989, 19-
27). After incubation for 24 to 48 hours at 37°C, the blue-
colored products of GUS activity were visible, and the
number of blue areas (i.e., blue cells indicating GUS
expression) was counted under a stereo microscope. Approx.
30% of the treated callus clumps showed one or several blue
areas (Kochihibiki: 12 of 41 electroporated callus clumps;
Chiyonishiki: 10 of 35 electroporated callus clumps).

Example 5: Establishment and maintenance of rice cultures of suspended cell clumps.

Immature zygotic embryos were isolated from surface
sterilized developing rice kernels (in milky stage) of
greenhouse-grown plants of the rice cultivars Kochihibiki
and Chiyonishiki. The immature embryos were placed, embryo
axis towards the medium, on solid 2N6 medium (see Example
1). The plated explants were kept at 27°C in darkness.
Within 2-3 weeks after culture initiation, small compact
yellowish callus clumps with a smooth surface grew out from
primary callus and directly from the explant. Approximately
30 of such callus clumps of different size, with diameters
of between 0.1 mm and 2.0 mm, were each transferred into
65 ml of N6 liquid medium (Chu et al (1975) supra),
supplemented with 0.3 g/l casamino acid (vitamin assay), 30
g/l sucrose and 1 mg/l 2,4-D, in a 250 ml Erlenmeyer flask.
These flasks with the cultures were kept in the dark on a
rotary shaker at approximately 120 rpm. Subculturing was
done weekly; at the first subculture (one week after
culture initiation), soft and whitish callus pieces were
discarded, and all medium was removed and replaced by 65 ml
of fresh N6 liquid medium. The same procedure was used at
the s cond subculture (2 we ks after culture initiation).
During the first tw w eks, the riginal callus clumps gr w

into bigger pieces. In the third week of culture, newly developed small cell aggregates started to separate from the bigger callus clumps. At the third subculture, these smaller aggregates and the big yellow compact pieces were selected and transferred to fresh liquid N6 medium At the fourth subculture, only small compact yellow clumps were selected and transferred to fresh medium; the bigger clumps were discarded. At the following subcultures, 1-2 ml packed cell volume (PVC) of selected small compact yellow aggregates were tranferred to 65 ml fresh N6 medium.

Example 6: Tranformation of rice cultures of suspended cell clumps

The cultures of suspended cell clumps from rice cultivars Kochihibiki and Chiyonishiki of Example 5 were transformed with a herbicide resistance gene, and transformed cells were regenerated into transgenic plants as follow:

1.   Kochihibiki

A culture of suspended cell clumps was established and maintained in N6 medium for 18 days prior to electroporation. Four days after the second weekly subculture, the N6 medium was removed, the cell clumps were washed with electroporation buffer 9 and then kept in this buffer for 1 hour at room temperature. The cell clumps were washed in buffer 9 again and transferred to electroporation cuvettes and resuspended in approximately 120 μl of buffer 9. About 13 μg of pDE110 DNA, linearized with HindIII, was added per cuvette, and the mixture of cell aggregates in buffer 9 and DNA was incubated, first for 45 min at room temperature and then for 10 min on ice. Then, a single pulse with a field strength of 650 V/cm was discharged from a 900 μF capacitor to th mixture of cell clumps and DNA.

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Liquid N67 medium was then added to the cuvettes, and the electroporated cell clumps were transfered to solid N67 medium supplemented with 5 mg/l PPT. After 23 days, the developing calli were transferred to fresh N67 medium plus PPT. After another 21 days on selective N67 medium, the selected calli were transferred to selective regeneration medium. After 35 days on regeneration medium, shoots were transferred to N6 hormone-free medium. For each callus, three in vitro plantlets of approximately 10 cm height were transferred to soil and to the greehouse.

In similar experiments, cultures of suspended cell aggregates were electroporated 6 days or 12 days after establishing the culture. From these experiments, transformed calli were obtained, from which transgenic plants were regenerated.

2. Chiyonishiki

A culture of suspended cell clumps was established and maintained using essentially the same procedures as described above for Kochihibiki in Example 6.1. However, cell clumps for electroporation were harvested four days after the third weekly subculture. Electroporation, subsequent callus initiation and propagation, and regeneration of plants were also carried out essentially as described in Example 6.1. Some of the cell clumps were electroporated with pDE110, and other cell clumps were electroporated with plasmid DNA that contained both a chimeric bar gene and another chimaeric gene containing the

DNA coding for barstar under the control of one of the following stamen-specific promoters of rice: PT72, PT42 and PE1 (WO 92/00274).

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Example 7: Analysis of transgenic plants of Example 6

Six Kochihibiki plants of Example 6.1, derived from two independent transgenic callus lines, were cultivated in the greenhouse, were sprayed with Basta, and were found to be Basta-resistant.

Two plants, designated as KB25 and KB28, each of which was derived from a different transgenic callus line, were analyzed in detail. Both plants scored positive in enzymatic assays for phosphinotricin acetyl transferase (PAT) activity. For Southern analysis, the rice genomic DNA was digested with the restriction enzymes XhoI, BglII and PvuII, Southern blotted and probed with pDE110 DNA. In both plants, the pDE110-derived DNA was found to be located on a single XhoI restriction fragment. KB25 and KB28 both contained a functional 35S promoter linked to the complete bar gene as indicated by the 1.54 kb BglII fragment and the 1.65 kb PvuII fragment. KB25 carried multiple inserts, and KB28 carried 1 to 4 copies of pDE110-derived DNA. Both plants were found to set seed.

Plants regenerated from a selected callus of Example 6.2, after transformation with pDE110, were cultivated in the greenhouse, sprayed with Basta, and were found to be Basta-resistant. One plant, designated as CB23, scored positive in a PAT assay and was found to contain 1-3 almost complete copies of pDE110 DNA in a Southern hybridization performed as described above for Kochihibiki.

Plants regenerated from selected calli of Example 6.2, after transformation with DNA containing two chimaeric genes, including the barstar gene, were found to be Basta-resistant, to be PAT-positive, and to c ntain b th the chimaeric bar gen and th chimaeric barstar g n .

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Expression of the barstar gene in immature spikelets is
determined by Northern analysis.

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SEQUENCE LISTING

1. General Information

i)   APPLICANT : PLANT GENETIC SYSTEMS N.V.

ii)   TITLE OF INVENTION : Transformation of monocot cells

iii) NUMBER OF SEQUENCES : 2

iv)   CORRESPONDENCE ADDRESS :
        A. ADDRESSEE : Plant Genetic Systems N.V.
        B. STREET : Plateaustraat 22,
        C. POSTAL CODE AND CITY : 9000 Ghent,
        D. COUNTRY : Belgium

v)   COMPUTER READABLE FORM :
        A. MEDIUM TYPE 5.25 inch, double sided, high density
        1.2 Mb floppy disk
        B. COMPUTER : IBM PC/AT
        C. OPERATING SYSTEM : DOS version 3.3
        D. SOFTWARE : WordPerfect

vi)   CURRENT APPLICATION DATA : Not Available

(vii)   PRIOR APPLICATION DATA : : Not applicable

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CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC ATTCGCCATT    250
CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT    300
TACGCCAGCT GGCGAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGGTA    350
ACGCCAGGGT TTTCCCAGTC ACGACGTTGT AAAACGACGG CCAGTGAATT    400
CCAATCCCAC CAAAACCTGA ACCTAGCAGT TCAGTTGCTC CTCTCAGAGA    450
CGAATCGGGT ATTCAACACC CTCATACCAA CTACTACGTC GTGTATAACG    500
GACCTCATGC CGGTATATAC GATGACTGGG GTTGTACAAA GGCAGCAACA    550
AACGGTGTTC CCGGAGTTGC GCATAAGAAG TTTGCCACTA TTACAGAGGC    600
AAGAGCAGCA GCTGACGCGT ATACAACAAG TCAGCAAACA GATAGGTTGA    650
ACTTCATCCC CAAAGGAGAA GCTCAACTCA AGCCCAAGAG CTTTGCGAAG    700
GCCCTAACAA GCCCACCAAA GCAAAAGCC CACTGCTCAC GCTAGGAACC    750
AAAAGGCCCA GCAGTGATCC AGCCCCAAAA GAGATCTCCT TTGCCCCGGA    800
GATTACAATG GACGATTTCC TCTATCTTTA CGATCTAGGA AGGAAGTTCG    850
AAGGTGAAGG TGACGACACT ATGTTCACCA CTGATAATGA GAAGGTTAGC    900
CTCTTCAATT TCAGAAAGAA TGCTGACCCA CAGATGGTTA GAGAGGCCTA    950
CGCAGCAGGT CTCATCAAGA CGATCTACCC GAGTAACAAT CTCCAGGAGA   1000
TCAAATACCT TCCCAAGAAG GTTAAAGATG CAGTCAAAAG ATTCAGGACT   1050
AATTGCATCA AGAACACAGA GAAAGACATA TTTCTCAAGA TCAGAAGTAC   1100
TATTCCAGTA TGGACGATTC AAGGCTTGCT TCATAAACCA AGGCAAGTAA   1150
TAGAGATTGG AGTCTCTAAA AAGGTAGTTC CTACTGAATC TAAGGCCATG   1200
CATGGAGTCT AAGATTCAAA TCGAGGATCT AACAGAACTC GCCGTGAAGA   1250
CTGGCGAACA GTTCATACAG AGTCTTTTAC GACTCAATGA CAAGAAGAAA   1300
ATCTTCGTCA ACATGGTGGA GCACGACACT CTGGTCTACT CCAAAAATGT   1350
CAAAGATACA GTCTCAGAAG ACCAAAGGGC TATTGAGACT TTTCAACAAA   1400
GGATAATTTC GGGAAACCTC CTCGGATTCC ATTGCCCAGC TATCTGTCAC   1450
TTCATCGAAA GGACAGTAGA AAAGGAAGGT GGCTCCTACA AATGCCATCA   1500
TTGCGATAAA GGAAAGGCTA TCATTCAAGA TGCCTCTGCC GACAGTGGTC   1550
CCAAAGATGG ACCCCCACCC ACGAGGAGCA TCGTGGAAAA AGAAGACGTT   1600
CCAACCACGT CTTCAAAGCA AGTGGATTGA TGTGACATCT CCACTGACGT   1650
AAGGGATGAC GCACAATCCC ACTATCCTTC GCAAGACCCT CCTCTATAT   1700
AAGGAAGTTC ATTTCATTTG GAGAGGACAC GCTGAAATCA CCAGTCTCTC   1750
TCTATAAATC TATCTCTCTC TCTATAACCA TGGACCCAGA ACGACGCCCG   1800
GCCGACATCC GCCGTGCCAC CGAGGCGGAC ATGCCGGCGG TCTGCACCAT   1850
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2. Information for SEQ ID NO. 1

i)   Sequence characteristics
            A. TYPE: nucleic acid
            B. LENGTH: 4883 bp
            C. STRANDEDNESS: double stranded
            D. TOPOLOGY: circular

ii)   MOLECULAR TYPE: pDE110 : plasmid DNA replicable in E.coli

ix)   Features:
            1 - 395 :   pUC18 derived sequence
            396 - 1779:   "3583" promoter sequence derived from
                             Cauliflower   mosaic   virus   isolate
                             CabbB-JI
            1780 - 2331:   coding   sequence   of   phosphinotricin
                             acetyltransferase gene
            2332 - 2619:   3' regulatory sequence containing the
                             polyadenylation site derived from
                             Agrobacterium T-DNA nopaline synthase
                             gene
            2620 - 4883:   pUC18 derived sequence

            Other information: plasmid is replicable in E.coli,
            confers ampicillin resistance to the bacterium

  xi)   Sequence description

        TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG   50
        GAGACGGTCA CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG   100
        TCAGGGCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG CTTAACTATG   150
        CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG GTGTGAAATA   200

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CGTCAACCAC TACATCGAGA CAAGCACGGT CAACTTCCGT ACCGAGCCGC    1900
AGGAACCGCA GGAGTGGACG GACGACCTCG TCCGTCTGCG GGAGCGCTAT    1950
CCCTGGCTCG TCGCCGAGGT GGACGGCGAG GTCGCCGGCA TCGCCTACGC    2000
GGGCCCCTGG AAGGCACGCA ACGCCTACGA CTGGACGGCC GAGTCGACCG    2050
TGTACGTCTC CCCCCGCCAC CAGCGGACGG GACTGGGCTC CACGCTCTAC    2100
ACCCACCTGC TGAAGTCCCT GGAGGCACAG GGCTTCAAGA GCGTGGTCGC    2150
TGTCATCGGG CTGCCCAACG ACCCGAGCGT GCGCATGCAC GAGGCGCTCG    2200
GATATGCCCC CCGCGGCATG CTGCGGGCGG CCGGCTTCAA GCACGGGAAC    2250
TGGCATGACG TGGGTTTCTG GCAGCTGGAC TTCAGCCTGC CGGTACCGCC    2300
CCGTCCGGTC CTGCCCGTCA CCGAGATCTG ATCTCACGCG TCTAGGATCC    2350
GAAGCAGATC GTTCAAACAT TTGGCAATAA AGTTTCTTAA GATTGAATCC    2400
TGTTGCCGGT CTTGCGATGA TTATCATATA ATTTCTGTTG AATTACGTTA    2450
AGCATGTAAT AATTAACATG TAATGCATGA CGTTATTTAT GAGATGGGTT    2500
TTTATGATTA GAGTCCCGCA ATTATACATT TAATACGCGA TAGAAAACAA    2550
AATATAGCGC GCAAACTAGG ATAAATTATC GCGCGCGGTG TCATCTATGT    2600
TACTAGATCG GGAAGATCCT CTAGAGTCGA CCTGCAGGCA TGCAAGCTTG    2650
GCGTAATCAT GGTCATAGCT GTTTCCTGTG TGAAATTGTT ATCCGCTCAC    2700
AATTCCACAC AACATACGAG CCGGAAGCAT AAAGTGTAAA GCCTGGGGTG    2750
CCTAATGAGT GAGCTAACTC ACATTAATTG CGTTGCGCTC ACTGCCCGCT    2800
TTCCAGTCGG GAAACCTGTC GTGCCAGCTG CATTAATGAA TCGGCCAACG    2850
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CTGACTCGCT GCGCTCGGTC GTTCGGCTGC GGCGAGCGGT ATCAGCTCAC    2950
TCAAAGGCGG TAATACGGTT ATCCACAGAA TCAGGGGATA ACGCAGGAAA    3000
GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT AAAAAGGCCG    3050
CGTTGCTGGC GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA    3100
AATCGACGCT CAAGTCAGAG GTGGCGAAAC CGACAGGAC TATAAAGATA     3150
CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT GCGCTCTCCT GTTCCGACCC    3200
TGCCGCTTAC CGGATACCTG TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG    3250
CTTTCTCAAT GCTCACGCTG TAGGTATCTC AGTTCGGTGT AGGTCGTTCG    3300
CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG    3350
CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA    3400
TCGCCACTGG CAGCAGCCAC TGGTAACAGG ATTAGCAGAG CGAGGTATGT    3450
AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC GGCTACACTA    3500
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GAAGGACAGT ATTTGGTATC TGCGCTCTGC TGAAGCCAGT TACCTTCGGA    3550
AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG    3600
TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC    3650
AAGAAGATCC TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA    3700
AACTCACGTT AAGGGATTTT GGTCATGAGA TTATCAAAAA GGATCTTCAC    3750
CTAGATCCTT TTAAATTAAA AATGAAGTTT TAAATCAATC TAAAGTATAT    3800
ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG TGAGGCACCT    3850
ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT GACTCCCCGT    3900
CGTGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG    3950
CAATGATACC GCGAGACCCA CGCTCACCGG CTCCAGATTT ATCAGCAATA    4000
AACCAGCCAG CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACTTTATC    4050
CGCCTCCATC CAGTCTATTA ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT    4100
CGCCAGTTAA TAGTTTGCGC AACGTTGTTG CCATTGCTAC AGGCATCGTG    4150
GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCAGCTCCG GTTCCCAACG    4200
ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT    4250
CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA    4300
CTCATGGTTA TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT    4350
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AGTGTATGCG GCGACCGAGT TGCTCTTGCC CGGCGTCAAT ACGGGATAAT    4450
ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG GAAAACGTTC    4500
TTCGGGGCGA AAACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA    4550
TGTAACCCAC TCGTGCACCC AACTGATCTT CAGCATCTTT TACTTTCACC    4600
AGCGTTTCTG GGTGAGCAAA AACAGGAAGG CAAAATGCCG CAAAAAAGGG    4650
AATAAGGGCG ACACGGAAAT GTTGAATACT CATACTCTTC CTTTTTCAAT    4700
ATTATTGAAG CATTTATCAG GGTTATTGTC TCATGAGCGG ATACATATTT    4750
GAATGTATTT AGAAAAATAA ACAAATAGGG GTTCCGCGCA CATTTCCCCG    4800
AAAAGTGCCA CCTGACGTCT AAGAAACCAT TATTATCATG ACATTAACCT    4850
ATAAAAATAG GCGTATCACG AGGCCCTTTC GTC                      4883
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3. Information for SEQ ID NO. 2

i)   Sequence characteristics
        A. TYPE: nucleic acid
        B. LENGTH: 5642 bp
        C. STRANDEDNESS: double stranded
        D. TOPOLOGY: circular

ii)   MOLECULAR TYPE: pDE4 : plasmid DNA replicable in E.coli

ix)   Features:
        1 - 395 :   pUC18 derived sequence
        396 - 1284:   "35S3" promoter sequence derived from
                        Cauliflower   mosaic   virus   isolate
                        CabbB-JI
        1285 - 3093:   coding sequence of ß-glucuronidase gene
        3094 - 3378:   3' regulatory sequence containing the
                        polyadenylation   site   derived   from
                        Agrobacterium T-DNA nopaline synthase
                        gene
        3379 - 5642:   pUC18 derived sequence

        Other information: plasmid is replicable in E.coli,
        confers ampicillin resistance to the bacterium

xi)   Sequence description

    TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG   50
    GAGACGGTCA CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG   100
    TCAGGGCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG CTTAACTATG   150
    CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG GTGTGAAATA   200
    CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC ATTCGCCATT   250
    CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT   300

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TACGCCAGCT  GGCGAAAGGG  GGATGTGCTG  CAAGGCGATT  AAGTTGGGTA     350
ACGCCAGGGT  TTTCCCAGTC  ACGACGTTGT  AAAACGACGG  CCAGTGAATT     400
CGAGCTCGGT  ACCCGGGGAT  CCTCTAGAGT  CGACCTGCAG  GCATGCAAGC     450
TCCTACGCAG  CAGGTCTCAT  CAAGACGATC  TACCCGAGTA  ACAATCTCCA     500
GGAGATCAAA  TACCTTCCCA  AGAAGGTTAA  AGATGCAGTC  AAAAGATTCA     550
GGACTAATTG  CATCAAGAAC  ACAGAGAAAG  ACATATTTCT  CAAGATCAGA     600
AGTACTATTC  CAGTATGGAC  GATTCAAGGC  TTGCTTCATA  AACCAAGGCA     650
AGTAATAGAG  ATTGGAGTCT  CTAAAAAGGT  AGTTCCTACT  GAATCTAAGG     700
CCATGCATGG  AGTCTAAGAT  TCAAATCGAG  GATCTAACAG  AACTCGCCGT     750
GAAGACTGGC  GAACAGTTCA  TACAGAGTCT  TTTACGACTC  AATGACAAGA     800
AGAAAATCTT  CGTCAACATG  GTGGAGCACG  ACACTCTGGT  CTACTCCAAA     850
AATGTCAAAG  ATACAGTCTC  AGAAGACCAA  AGGGCTATTG  AGACTTTTCA     900
ACAAAGGATA  ATTTCGGGAA  ACCTCCTCGG  ATTCCATTGC  CCAGCTATCT     950
GTCACTTCAT  CGAAAGGACA  GTAGAAAAGG  AAGGTGGCTC  CTACAAATGC    1000
CATCATTGCG  ATAAAGGAAA  GGCTATCATT  CAAGATGCCT  CTGCCGACAG    1050
TGGTCCCAAA  GATGGACCCC  CACCCACGAG  GAGCATCGTG  GAAAAAGAAG    1100
ACGTTCCAAC  CACGTCTTCA  AAGCAAGTGG  ATTGATGTGA  CATCTCCACT    1150
GACGTAAGGG  ATGACGCACA  ATCCCACTAT  CCTTCGCAAG  ACCCTTCCTC    1200
TATATAAGGA  AGTTCATTTC  ATTTGGAGAG  GACACGCTGA  AATCACCAGT    1250
CTCTCTCTAT  AAATCTATCT  CTCTCTCTAT  AACCATGGTC  CGTCCTGTAG    1300
AAACCCCAAC  CCGTGAAATC  AAAAAACTCG  ACGGCCTGTG  GCATTCAGT     1350
CTGGATCGCG  AAAACTGTGG  AATTGATCAG  CGTTGGTGGG  AAAGCGCGTT    1400
ACAAGAAAGC  CGGGCAATTG  CTGTGCCAGG  CAGTTTTAAC  GATCAGTTCG    1450
CCGATGCAGA  TATTCGTAAT  TATGCGGGCA  ACGTCTGGTA  TCAGCGCGAA    1500
GTCTTTATAC  CGAAAGGTTG  GGCAGGCCAG  CGTATCGTGC  TGCGTTTCGA    1550
TGCGGTCACT  CATTACGGCA  AAGTGTGGGT  CAATAATCAG  GAAGTGATGG    1600
AGCATCAGGG  CGGCTATACG  CCATTTGAAG  CCGATGTCAC  GCCGTATGTT    1650
ATTGCCGGGA  AAAGTGTACG  TATCACCGTT  TGTGTGAACA  ACGAACTGAA    1700
CTGGCAGACT  ATCCCGCCGG  GAATGGTGAT  TACCGACGAA  AACGGCAAGA    1750
AAAAGCAGTC  TTACTTCCAT  GATTTCTTTA  ACTATGCCGG  AATCCATCGC    1800
AGCGTAATGC  TCTACACCAC  GCCGAACACC  TGGGTGGACG  ATATCACCGT    1850
GGTGACGCAT  GTCGCGCAAG  ACTGTAACCA  CGCGTCTGTT  GACTGGCAGG    1900
TGGTGGCCAA  TGGTGATGTC  AGCGTTGAAC  TGCGTGATGC  GGATCAACAG    1950
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GTGGTTGCAA CTGGACAAGG CACTAGCGGG ACTTTGCAAG TGGTGAATCC    2000
GCACCTCTGG CAACCGGGTG AAGGTTATCT CTATGAACTG TGCGTCACAG    2050
CCAAAAGCCA GACAGAGTGT GATATCTACC CGCTTCGCGT CGGCATCCGG    2100
TCAGTGGCAG TGAAGGGCGA ACAGTTCCTG ATTAACCACA AACCGTTCTA    2150
CTTTACTGGC TTTGGTCGTC ATGAAGATGC GGACTTACGT GGCAAAGGAT    2200
TCGATAACGT GCTGATGGTG CACGACCACG CATTAATGGA CTGGATTGGG    2250
GCCAACTCCT ACCGTACCTC GCATTACCCT TACGCTGAAG AGATGCTCGA    2300
CTGGGCAGAT GAACATGGCA TCGTGGTGAT TGATGAAACT GCTGCTGTCG    2350
GCTTTAACCT CTCTTTAGGC ATTGGTTTCG AAGCGGGCAA CAAGCCGAAA    2400
GAACTGTACA GCGAAGAGGC AGTCAACGGG GAAACTCAGC AAGCGCACTT    2450
ACAGGCGATT AAAGAGCTGA TAGCGCGTGA CAAAAACCAC CCAAGCGTGG    2500
TGATGTGGAG TATTGCCAAC GAACCGGATA CCCGTCCGCA AGTGCACGGG    2550
AATATTTCGC CACTGGCGGA AGCAACGCGT AAACTCGACC CGACGCGTCC    2600
GATCACCTGC GTCAATGTAA TGTTCTGCGA CGCTCACACC GATACCATCA    2650
GCGATCTCTT TGATGTGCTG TGCCTGAACC GTTATTACGG ATGGTATGTC    2700
CAAAGCGGCG ATTTGGAAAC GGCAGAGAAG GTACTGGAAA AAGAACTTCT    2750
GGCCTGGCAG GAGAAACTGC ATCAGCCGAT TATCATCACC GAATACGGCG    2800
TGGATACGTT AGCCGGGCTG CACTCAATGT ACACCGACAT GTGGAGTGAA    2850
GAGTATCAGT GTGCATGGCT GGATATGTAT CACCGCGTCT TTGATCGCGT    2900
CAGCGCCGTC GTCGGTGAAC AGGTATGGAA TTTCGCCGAT TTTGCGACCT    2950
CGCAAGGCAT ATTGCGCGTT GGCGGTAACA AGAAAGGGAT CTTCACTCGC    3000
GACCGCAAAC CGAAGTCGGC GGCTTTTCTG CTGCAAAAAC GCTGGACTGG    3050
CATGAACTTC GGTGAAAAAC CGCAGCAGGG AGGCAAACAA TGAXXXXXG    3100
AATTGATCCG AAGCAGATCG TTCAAACATT TGGCAATAAA GTTTCTTAAG    3150
ATTGAATCCT GTTGCCGGTC TTGCGATGAT TATCATATAA TTTCTGTTGA    3200
ATTACGTTAA GCATGTAATA ATTAACATGT AATGCATGAC GTTATTTATG    3250
AGATGGGTTT TTATGATTAG AGTCCCGCAA TTATACATTT AATACGCGAT    3300
AGAAAACAAA ATATAGCGCG CAAACTAGGA TAAATTATCG CGCGCGGTGT    3350
CATCTATGTT ACTAGATCGG GAAGATCCTC TAGAGTCGAC CTGCAGGCAT    3400
GCAAGCTTGG CGTAATCATG GTCATAGCTG TTTCCTGTGT GAAATTGTTA    3450
TCCGCTCACA ATTCCACACA ACATACGAGC CGGAAGCATA AAGTGTAAAG    3500
CCTGGGGTGC CTAATGAGTG AGCTAACTCA CATTAATTGC GTTGCGCTCA    3550
CTGCCCGCTT TCCAGTCGGG AAACCTGTCG TGCCAGCTGC ATTAATGAAT    3600
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CGGCCAACGC GCGGGGAGAG GCGGTTTGCG TATTGGGCGC TCTTCCGCTT    3650
CCTCGCTCAC TGACTCGCTG CGCTCGGTCG TTCGGCTGCG GCGAGCGGTA    3700
TCAGCTCACT CAAAGGCGGT AATACGGTTA TCCACAGAAT CAGGGGATAA    3750
CGCAGGAAAG AACATGTGAG CAAAAGGCCA GCAAAAGGCC AGGAACCGTA    3800
AAAAGGCCGC GTTGCTGGCG TTTTTCCATA GGCTCCGCCC CCCTGACGAG    3850
CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC CGACAGGACT    3900
ATAAAGATAC CAGGCGTTTC CCCCTGGAAG CTCCCTCGTG CGCTCTCCTG    3950
TTCCGACCCT GCCGCTTACC GGATACCTGT CCGCCTTTCT CCCTTCGGGA    4000
AGCGTGGCGC TTTCTCAATG CTCACGCTGT AGGTATCTCA GTTCGGTGTA    4050
GGTCGTTCGC TCCAAGCTGG GCTGTGTGCA CGAACCCCCC GTTCAGCCCG    4100
ACCGCTGCGC CTTATCCGGT AACTATCGTC TTGAGTCCAA CCCGGTAAGA    4150
CACGACTTAT CGCCACTGGC AGCAGCCACT GGTAACAGGA TTAGCAGAGC    4200
GAGGTATGTA GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG CCTAACTACG    4250
GCTACACTAG AAGGACAGTA TTTGGTATCT GCGCTCTGCT GAAGCCAGTT    4300
ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC AAACCACCGC    4350
TGGTAGCGGT GGTTTTTTTG TTTGCAAGCA GCAGATTACG CGCAGAAAAA    4400
AAGGATCTCA AGAAGATCCT TTGATCTTTT CTACGGGGTC TGACGCTCAG    4450
TGGAACGAAA ACTCACGTTA AGGGATTTTG GTCATGAGAT TATCAAAAAG    4500
GATCTTCACC TAGATCCTTT TAAATTAAAA ATGAAGTTTT AAATCAATCT    4550
AAAGTATATA TGAGTAAACT TGGTCTGACA GTTACCAATG CTTAATCAGT    4600
GAGGCACCTA TCTCAGCGAT CTGTCTATTT CGTTCATCCA TAGTTGCCTG    4650
ACTCCCCGTC GTGTAGATAA CTACGATACG GGAGGGCTTA CCATCTGGCC    4700
CCAGTGCTGC AATGATACCG CGAGACCCAC GCTCACCGGC TCCAGATTTA    4750
TCAGCAATAA ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA GTGGTCCTGC    4800
AACTTTATCC GCCTCCATCC AGTCTATTAA TTGTTGCCGG GAAGCTAGAG    4850
TAAGTAGTTC GCCAGTTAAT AGTTTGCGCA ACGTTGTTGC CATTGCTACA    4900
GGCATCGTGG TGTCACGCTC GTCGTTTGGT ATGGCTTCAT TCAGCTCCGG    4950
TTCCCAACGA TCAAGGCGAG TTACATGATC CCCCATGTTG TGCAAAAAAG    5000
CGGTTAGCTC CTTCGGTCCT CCGATCGTTG TCAGAAGTAA GTTGGCCGCA    5050
GTGTTATCAC TCATGGTTAT GGCAGCACTG CATAATTCTC TTACTGTCAT    5100
GCCATCCGTA AGATGCTTTT CTGTGACTGG TGAGTACTCA ACCAAGTCAT    5150
TCTGAGAATA GTGTATGCGG CGACCGAGTT GCTCTTGCCC GGCGTCAATA    5200
CGGGATAATA CCGCGCCACA TAGCAGAACT TTAAAAGTGC TCATCATTGG    5250
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AAAACGTTCT TCGGGGCGAA AACTCTCAAG GATCTTACCG CTGTTGAGAT      5300
CCAGTTCGAT GTAACCCACT CGTGCACCCA ACTGATCTTC AGCATCTTTT      5350
ACTTTCACCA GCGTTTCTGG GTGAGCAAAA ACAGGAAGGC AAAATGCCGC      5400
AAAAAAGGGA ATAAGGGCGA CACGGAAATG TTGAATACTC ATACTCTTCC      5450
TTTTTCAATA TTATTGAAGC ATTTATCAGG GTTATTGTCT CATGAGCGGA      5500
TACATATTTG AATGTATTTA GAAAAATAAA CAAATAGGGG TTCCGCGCAC      5550
ATTTCCCCGA AAAGTGCCAC CTGACGTCTA AGAAACCATT ATTATCATGA      5600
CATTAACCTA TAAAAATAGG CGTATCACGA GGCCCTTTCG TC              5642
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<u>CLAIMS</u>

1. A process for genetically transforming a genome, particularly a nuclear genome, of a cell of a monocotyledonous plant, particularly a gramineous plant, comprising the step of:

      electroporating, with a DNA fragment, cells of said plant, preferably an aggregate of said cells; each of said cells possessing at least part of its plant cell wall and forming part of, or being capable of forming, a culture of suspended cells, such as a cell suspension culture.

2. The process of claim 1 in which a culture of suspended cells is electroporated that is not older than four months, preferably not older than three months, particularly for suspended rice cells.

3. The process of claim 2 in which the majority of the cells, preferably at least 75%, particularly at least 80%, quite particularly at least 90%, of said culture of suspended cells, have a normal chromosome number.

4. The process of claims 2 or 3 in which said culture of suspended cells is a regenerable cell suspension culture, particularly an embryogenic cell suspension culture.

5. The process of any one of claims 1-4, comprising the additional steps of:

      regenerating, from said transformed cell, a transformed phenotypically normal plant.

6. The process of any one of claims 1 to 5 in which said

monocotyledonous plant is rice, particularly cultivars Nipponbare or Kochihibiki.

7. The process of any one of claims 2 to 6 in which said electroporation is carried out in a cuvette containing: 30 to 150 mg, preferably 75 to 100 mg, of cell aggregates of said culture of suspended cells; and 50 to 30µg, preferably 10 to 20 µg, of said DNA fragment, preferably in 100 to 200 µl of electroporation buffer.

8. The process of claim 7 in which the electroporation is carried out by discharging a single pulse from a capacitor with a voltage of between 600 to 700 V/cm and a capacitance of between 800 to 900 µF.

9. A transformed cell of a monocotyledonous plant, particularly a gramineous plant, quite particularly a cereal such as rice, wheat or barley, made by the process of any one of claims 1 to 8.

10. A transformed monocotyledonous plant, particularly a gramineous plant, quite particularly a cereal such as rice, wheat or barley, consisting essentially of cells of claim 9.

11. A seed of the plant of claim 10.

(51) International Patent Classification 5 :	A3	(11) International Publication Number:	WO 93/21335
C12N 15/87, 15/04, A01H 5/00		(43) International Publication Date:	28 October 1993 (28.10.93)

(54) Title: TRANSFORMATION OF MONOCOT CELLS

(57) Abstract

A process for genetically transforming the nuclear genome of a cell of a monocotyledonous plant, particularly a gramineous plant, comprising the step of: electroporating, with one or more DNA fragments, either a culture of suspended cells of the plant or cells of the plant capable of forming the culture, each cell possessing at least part of its plant cell wall.

INTERNATIONAL SEARCH REPORT

International Applicat   io   PCT/EP 93/00905

I. CLASSIFICATION OF SUBJECT MATTER   (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5   C 12 N   15/87   C 12 N   15/04   A 01 H   5/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 12 N A 01 H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[°]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	BIOLOGICAL ABSTRACTS, vol. 94, abstract no. 028273, Philadelphia, PA, US, J.-S. YANG et al.: "Introduction of foreign gene into small cell groups digested partial-enzymically in rice and regeneration of transgenic plants" & ACTA BOT SIN 1991, vol. 33, no. 11, pages 825-832 ---	1,3.5,9 ,10
Y	idem ---	11
X	SCIENCE IN CHINA SERIES B, vol. 34, no. 8, 1991, pages 923-931, B.-J. LI et al.: "Introduction of foreign genes into the seed embryo cells of rice by electroinjection and the regeneration of transgenic rice plants" * the whole document * --- -/-	1,2-5,9 ,10

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27-10-1993	2 1 -12- 1993
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	GURDJIAN

III. DOCUMENTS CONSIDERED TO BE RELEVANT	(CONTINUED FROM THE SECOND SHEET)	
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	idem ---	8
X	BIOLOGICAL ABSTRACTS, vol. 91, abstract no. 095740, Philadalphia, PA, US, H.-Q. XU et al.: "Gene transfer into intact plant cells by electroporation" & ACTA BOT SIN 1990, vol. 32, no. 10, pages 759-765 * abstract * ---	1,3,5,9,10
Y	EMBO JOURNAL, vol. 10, no. 7, 1991, pages 1803-1808, Eynsham, Oxford, GB, Y. TADA et al.: "Expression of a monocot LHCP promoter in transgenic rice" * the whole document * ---	11
A	idem ---	1,3-5,9,10
A	BIOLOGICAL ABSTRACTS, vol. 92, abstract no. 119547, Philadelphia, PA, US, S.J. HWANG et al.: "Isolation culture and electroporation of rice protoplasts" & KOREAN J BOT 1991, vol. 34, no. 1, pages 19-24 ---	1,4,8
A	EP,A,0414551 (MITSUI TOATSU CHEMICALS, INC.) 27.02.1991 * the whole document * ---	1,7,8
A	BIOLOGICAL ABSTRACTS, vol. 90, abstract no. 086872, Philadelphia, PA, US, A.HAYASHIMOTO et al.: "A polyethylene glycol-mediated protoplast transformation system for production of fertile transgenic rice plants" & PLANT PHYSIOL (BETHESDA) 1990, vol. 93, no. 3, pages 857-863 * abstract * ---	1,6
A	BIOLOGICAL ABSTRACTS, vol. 90, abstract no. 086860, Philadelphia, PA, US, R.A. DEKEYSER et al.: "Transient gene expression in intact and organized rice tissues" & PLANT CELL 1990, vol. 2, no. 7, pages 591-602 ---	1,9,10
A	DATABASE WPIL, Derwent Publications Ltd., London, GB, DATABASE WPIL, AN 87-323404 [46], & JP - A - 62228277 (KIRIN BREWERY KK) 1987 * abstract * -----	1-3,7,8